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Supplemental Information

Genetically Encoded Spy Peptide Fusion System to Detect Plasma Membrane-Localized Proteins In Vivo

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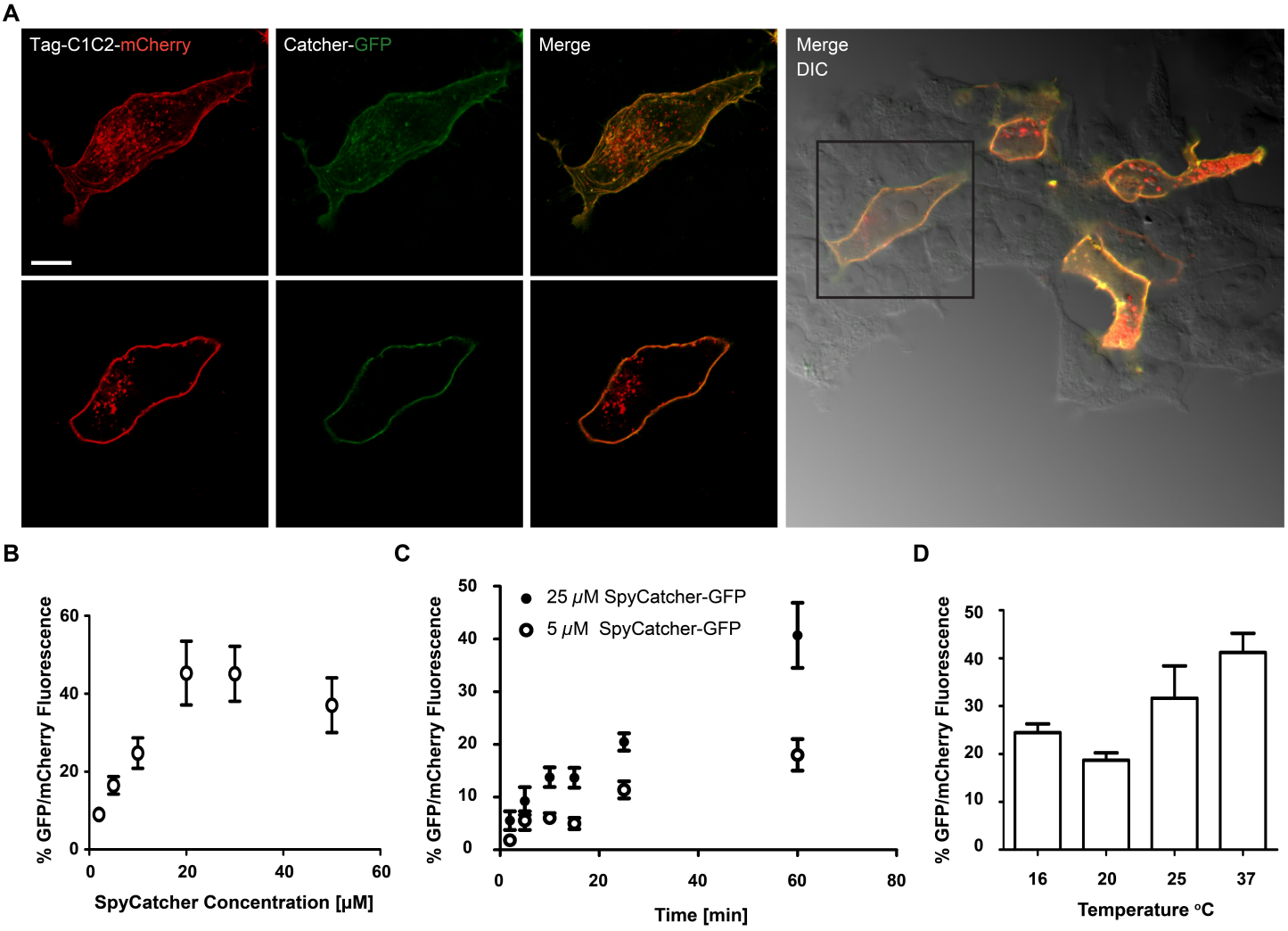

A
Tag-C1C2-mCherry
Catcher-GFP
Merge
Merge
DIC
B
% GFP/mCherry Fluorescence
SpyCatcher Concentration [μM]
C
% GFP/mCherry Fluorescence
25 μM SpyCatcher-GFP
5 μM SpyCatcher-GFP
Time [min]
D
% GFP/mCherry Fluorescence
Temperature °C

Figure S1, Related to Figure 1. Catcher-GFP labeling of membrane-localized Tag-C1C2-mCherry in live HEK cells and optimization of SpyTag/SpyCatcher binding efficiency in complex media used for mammalian cell cultures.

(**A**) Top row: (left) Maximum intensity projection of Tag-C1C2-mCherry expression in HEK cells (red), (middle left) Catcher-GFP membrane localized protein binding (green) and (middle right) a merge. Bottom row shows single plane confocal images of cell in each channel. (right) Single plane confocal image of a population of HEK cells with only a fraction of cells expressing Tag-C1C2-mCherry. Black box indicates cell shown to the left. Only the cells expressing the Tag-C1C2-mCherry show binding of the Catcher-GFP. (**B**) Effect of different concentrations of extracellular Catcher-GFP. Plot shows quantification of GFP fluorescence relative to mCherry fluorescence of individual labeled Tag-C1C2-mCherry expressing cells. Fluorescence measurements were obtained from single plane confocal images of Catcher-GFP bound to membrane-localized Tag-C1C2-mCherry after treatment of HEK cells with Catcher-GFP for 1 hour in D10 medium (*N* = 12-14 cells for each concentration). (**C**) Testing different incubation times from 2-60 min at 25^{o}C. Plot shows the percent of GFP fluorescence relative to mCherry fluorescence of individual Tag-C1C2-mCherry expressing cells covalently bound to Catcher-GFP after incubation of Tag-C1C2-mCherry expressing cells with either 5 μM (empty circles) or 25 μM (filled circles) Catcher-GFP (*N* = 11-14 for each time point). (**D**) Effect of temperature from 16 to 37 ^{o}C. Plot of the percent of GFP fluorescence relative to mCherry fluorescence of individual Tag-C1C2-mCherry expressing cells bound to Catcher-GFP after incubation of Tag-C1C2-mCherry expressing cells with 25 uM Catcher-GFP for 1 hour (*N* = 9-14 for each temp). All population data are plotted as mean ± SEM.

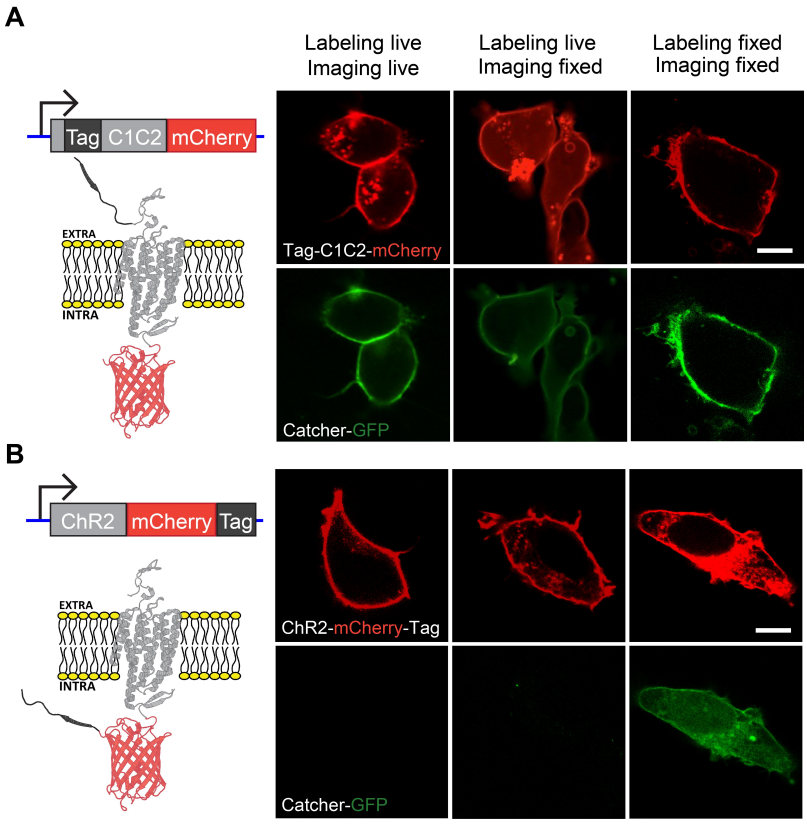

A
Labeling live
Imaging live
Labeling live
Imaging fixed
Labeling fixed
Imaging fixed
Tag
C1C2
mCherry
EXTRA
INTRA
Tag-C1C2-mCherry
Catcher-GFP
B
ChR2
mCherry
Tag
EXTRA
INTRA
ChR2-mCherry-Tag
Catcher-GFP

Figure S2, Related to Figure 2. SpyTag/SpyCatcher system works with both live and fixed cultured cells and can be used to identify the signal peptide of ChR2 and its positioning can affect ChR2 membrane localization.

(**A**) (left) Schematic of N-terminal SpyTagged opsin construct (Tag-C1C2-mCherry) in the cell membrane with the SpyTag displayed on the extracellular surface. (**B**) (left) Schematic of C-terminal SpyTagged opsin construct (ChR2-mCherry-Tag) in the cell membrane with the SpyTag displayed on the intracellular side of the cell. (**A**) & (**B**) (right) Single plane confocal images of the two opsin constructs with varying labeling and fixation methods. Column 1: both constructs show expression of the tagged ChR-mCherry. With extracellular application of Catcher-GFP only the N-terminal SpyTag shows Catcher-GFP binding since the Catcher-GFP cannot penetrate the membrane to label the C-terminal SpyTag. Column 2: fixation in paraformaldehyde (PFA) has no effect of the membrane-localized tagging after covalent binding of the Catcher-GFP. Column 3: fixation with PFA permeabilizes the cells allowing Catcher-GFP to get through the membrane and then covalently bind total ChR-mCherry for both the N-terminal and C-terminal SpyTagged constructs. Scale bar, 10 μm.

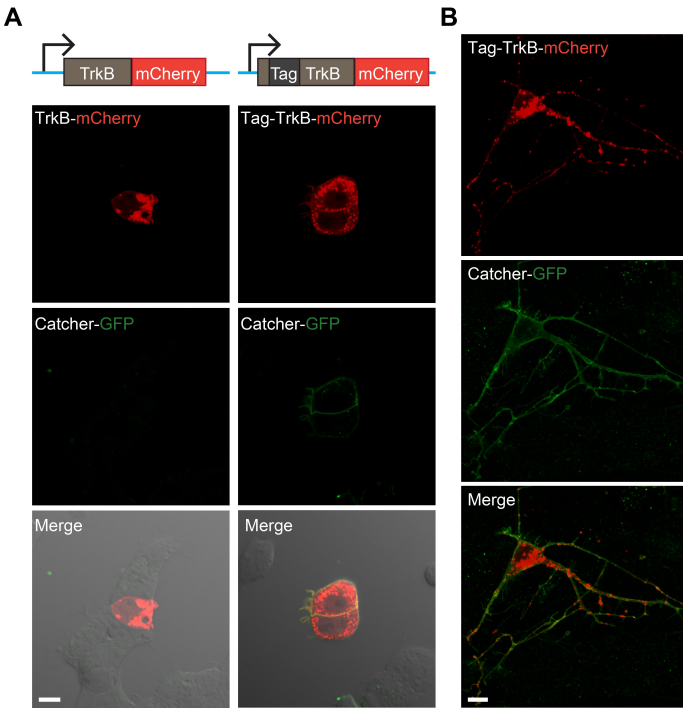

A
TrkB
mCherry
Tag
TrkB
mCherry
TrkB-mCherry
Tag-TrkB-mCherry
Catcher-GFP
Catcher-GFP
Merge
Merge
B
Tag-TrkB-mCherry
Catcher-GFP
Merge

Figure S3, Related to Figure 2. SpyTag/SpyCatcher labeling of TrkB receptor transfected in HEK cells and neurons.

(**A**) The SpyTag was placed at the N-terminus after the signal peptide cleavage site of the TrkB-mCherry fusion. Single plane confocal images of HEK cells expressing TrkB-mCherry and Tag-TrkB-mCherry (red) after 1-hour incubation with Catcher-GFP (green). Only the Tag-TrkB-mCherry expressing cells show labeling with Catcher-GFP. (**B**) Maximum intensity projection of the Tag-TrkB-mCherry expressed in primary neuronal cultures (red) labeled with Catcher-GFP (green). Scale bar, 10 μm.

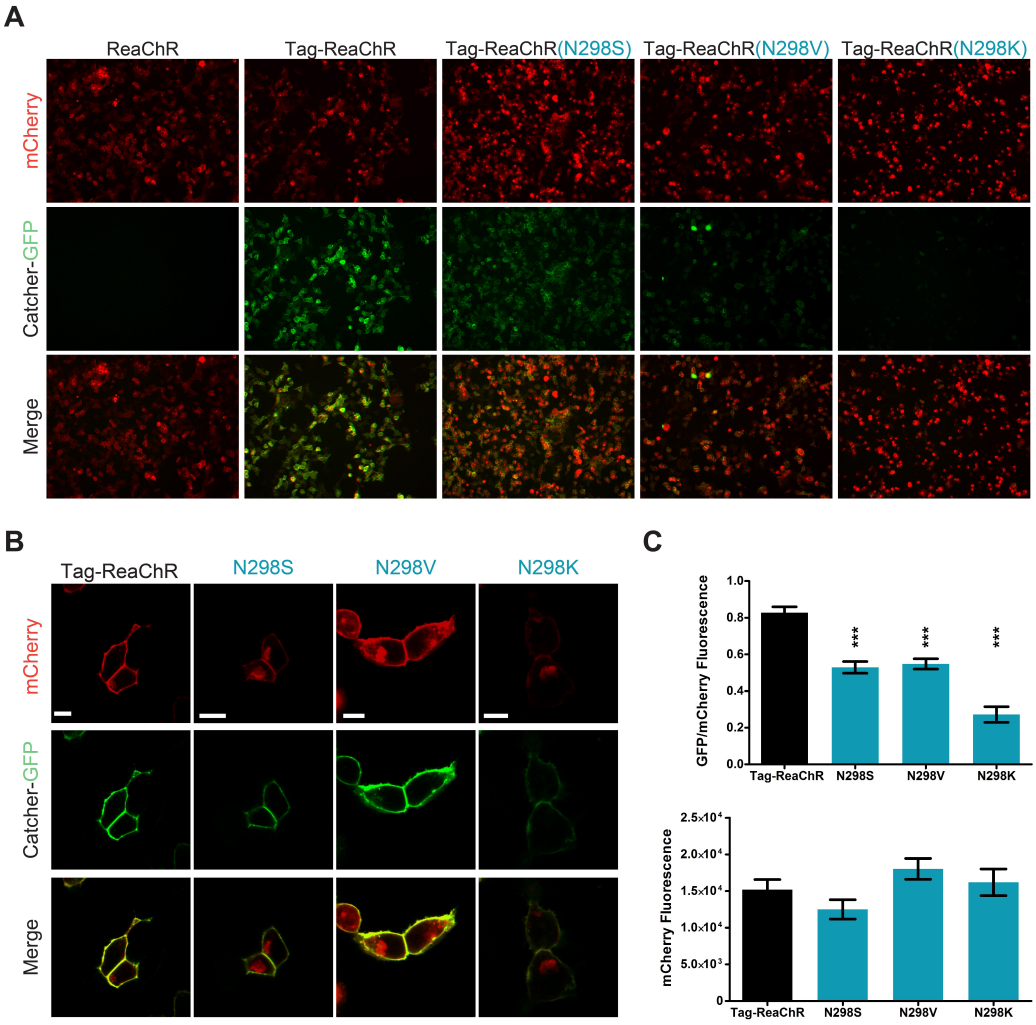
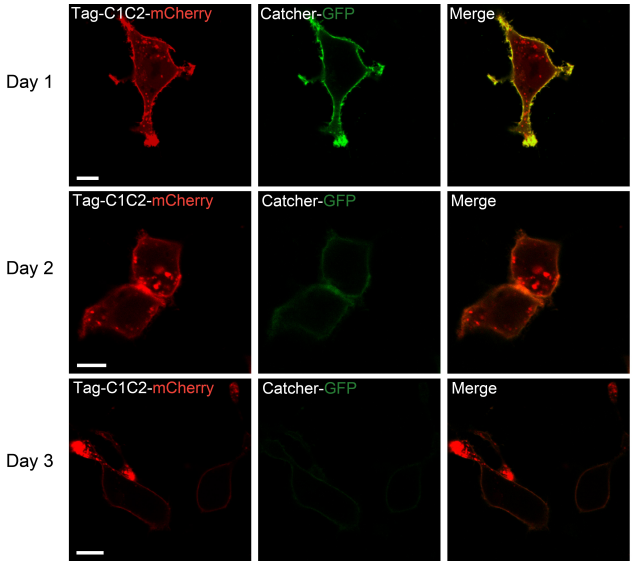

A
ReaChR
Tag-ReaChR
Tag-ReaChR(N298S)
Tag-ReaChR(N298V)
Tag-ReaChR(N298K)
mCherry
Catcher-GFP
Merge
B
Tag-ReaChR
N298S
N298V
N298K
mCherry
Catcher-GFP
Merge
C
GFP/mCherry Fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
Tag-ReaChR
N298S
N298V
N298K
mCherry Fluorescence
2.5x10⁴
2.0x10⁴
1.5x10⁴
1.0x10⁴
5.0x10³
0
Tag-ReaChR
N298S
N298V
N298K

Figure S4, Related to Figure 3. Characterization of a subset of variants with poor membrane localization identified in the SpyTag/SpyCatcher screen of the ReaChR N298 library.

(**A**) Example images from the screening process for non-tagged control (ReaChR), parent (Tag-ReaChR), and Tag-ReaChR mutant 'poor localizers' from the N298 library. Full field, population images were taken for each tested variant and used to measure the GFP and mCherry fluorescence. Amino acid mutations at the 298 residue position are highlighted in blue for the 'poor localizers' in the variants labeled as in Figure 3*D*. (**B**) Single plane confocal images of parent (Tag-ReaChR-mCherry) compared with the 'poor localizers' of mCherry (red), Catcher-GFP (green) and merge. All 'poor localizers' show high levels of internal mCherry localization. (**C**) (top) GFP/mCherry fluorescence ratio or (bottom) mCherry fluorescence of Tag-ReaChR (N = 24) compared with ReaChR variants (N298S: N = 44, N298V: N = 68, and N298K: N = 26) from single plane confocal images of HEK cells expressing the tagged opsins with intensity measurements made by selection of a region of interest around each cell and measurement of mean GFP and mCherry fluorescence across the region. Comparisons between Tag-ReaChR parent with each variant was done by Dunnett's Multiple Comparison Test. All population data are plotted as mean ± SEM. *P < 0.05, **P < 0.01, *** P < 0.001. Scale bar, 10 μm.


Day 1
Day 2
Day 3
Tag-C1C2-mCherry
Catcher-GFP
Merge
Tag-C1C2-mCherry
Catcher-GFP
Merge
Tag-C1C2-mCherry
Catcher-GFP
Merge

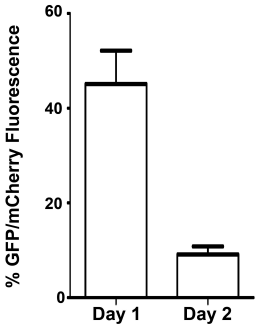
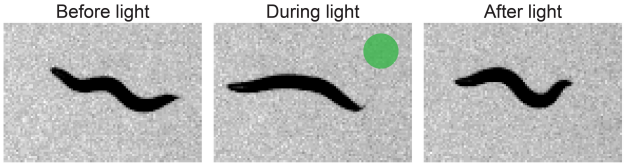

% GFP/mCherry Fluorescence
60
40
20
0
Day 1
Day 2

Figure S5, Related to Figure 5. Long-term stability of SpyTag/SpyCatcher labeling.

Single plane confocal images of Tag-C1C2-mCherry expression (red) and Catcher-GFP membrane-localized protein binding (green) and merge. Day 1 is imaged shortly after a 1-hour incubation of Catcher-GFP with HEK cells expressing Tag-C1C2-mCherry in D10 and washing with MEM. Cells were then left in D10 at 37^{o}C for 24 hours and imaged again for Tag-C1C2-mCherry expression (Day 2). Cells were then left in D10 at 37^{o}C for another 24 hours and imaged again for Tag-C1C2-mCherry expression (Day 3). (right) Plot of the percent of GFP/mCherry fluorescence of individual Tag-C1C2-mCherry expressing cells covalently bound to Catcher-GFP on Day 1 vs Day 2 (*N* = 12 for each day). All population data are plotted as mean ± SEM. Scale bar, 10 μm.

A



B

	Catcher-GFP labeling?	ATR?	Fraction worms showing light induced paralysis
Tag-ReaChR-mCherry	NO	YES	11/11
Tag-ReaChR-mCherry	YES	YES	6/6
Tag-ReaChR-mCherry	NO	NO	0/3
Tag-ReaChR(E130D)-mCherry	NO	YES	10/10

Figure S6, Related to Figure 6. Functional characterization of Tag-opsin constructs in locomotion behavioral assay in *C. elegans*.

(**A**) Three frames of video of a *C. elegans* expressing Tag-ReaChR-mCherry specifically in GABA-producing neurons (19 D-type neurons) before (left), during (middle) and after (right) green light stimulation. Activation of these GABA neurons paralyses the worm. Activation of Tag-ReaChR-mCherry with green light shows clear induction of paralysis as shown by the posture change evident during light stimulation. (**B**) Table showing the fraction of worms with high opsin expression that have light induced paralysis under different conditions.

Movie S1, Related to Figure 6. Light activation of *C. elegans* GABA-producing neurons expressing Tag-ReaChR-mCherry to inhibit body muscle contractions and cause paralysis of the animals.

Video shows moving worm under low red light illumination then upon green light exposure (indicated by bright light in video) the worm becomes paralyzed. Once the green light is turned off the worm immediately recovers and begins moving again.

Table S1, Related to Experimental Procedures. Comparison between size of SpyTag with other covalent labeling methods.

Tag Name	Size [amino acids]	Reference
SpyTag	13	(Zakeri et al., 2012)
SpyTag optimized	10	(Li et al., 2014)
SpyCatcher	139	(Zakeri et al., 2012)
SpyCatcher optimized	84	(Li et al., 2014)
SNAP-Tag	181	(Gronemeyer et al., 2006)
CLIP-Tag	181	(Gautier et al., 2008)
Halo Tag	295	(Los et al., 2008)
GFP	238	(Tsien, 1998)

Table S2, Related to Experimental Procedures. Summary of constructs built with protein product name used in the text.

Construct	Protein
pLenti-CMV/CaMKIIa::SpyTag-C1C2-TS-EYFP*	Tag-C1C2-EYFP
pLenti-CMV/CaMKIIa::SpyTag-C1C2-TS-mCherry*	Tag-C1C2-mCherry
plenti-CMV/CaMKIIa::SNAP-tag-C1C2-TS-mCherry*	SNAP-tag-C1C2-mCherry
pLenti-CMV/CaMKIIa::C1C2-TS-mCherry*	C1C2-mCherry
pQE80l-T5::6xhis-SpyCatcher-Elp-GFP	Catcher-GFP
pLenti-CMV/CaMKIIa::SpyTag(DA)-C1C1-TS-mCherry*	Tag(DA)-C1C1-mCherry
pLenti-CMV/CaMKIIa::SpyTag(0)-C1C1-TS-mCherry*	Tag^0-C1C1-mCherry
pGP-CMV::ChR2-mCherry	ChR2-mCherry
pGP-CMV::ChR2-mCherry-SpyTag	ChR2-mCherry-Tag
pLenti-CMV/CAMKIIa::ReaChR-TS-mCherry-WPRE*	ReaChR-mCherry
pLenti-CMV/CAMKIIa::SpyTag-ReaChR-TS-mCherry-WPRE*	Tag-ReaChR-mCherry
pLenti-CMV/hSyn1::TrkB-3xGS linker- mCherry-WPRE*	TrkB-mCherry
pLenti-CMV/hSyn1::SpyTag-TrkB-mCherry-WPRE*	Tag-TrkB-mCherry
hlh-12::SpyTag-C1C2-mCherry	Tag-C1C2-mCherry
HS::lin-3 signal sequence::SpyCatcher-GFP	Catcher-GFP
pSM*::unc-47::SpyTag-ReaChR-TS-mCherry*	Tag-ReaChR-mCherry
pSM*::unc-47::SpyTag-ReaChR(E130D)-TS-mCherry*	Tag-ReaChR(E130D)-mCherry

**Constructs denoted to have a CaMKIIa or hSyn promoter also have an upstream CMV promoter. The CMV promoter is responsible for expression in transfected cells while the CaMKIIa or hSyn promoter controls expression in virally infected cells. The work in this paper uses only transfected cells so the CMV promoter drives all reported mammalian cell expression.*

Table S3, Related to Experimental Procedures. Addgene plasmids with accession codes used for construct designs used in this paper.

Construct	Addgene #
pAAV-CaMKIIa-C1V1 (t/t)-TS-mCherry	35500
pLenti-CaMKIIa-C1C2-TS-EYFP[*]	35520
pLenti-CaMKIIa-hChR2(H134R)-mCherry-WPRE[*]	20943
pGP-CMV-GCaMP6f	40755
pLenti-hSyn-eNpHR 3.0-EYFP[*]	26775

[*]*Constructs denoted to have a CaMKII, CaMKIIa or hSyn promoter also have an upstream CMV promoter. The CMV promoter is responsible for expression in transfected cells while the CaMKIIa or hSyn promoter controls expression in virally infected cells. The work in this paper uses only transfected cells so the CMV promoter drives all reported mammalian cell expression.*

Table S4. Cloning primers used for construct design and construction.

Primer	Sequence (5' …. 3')	Used to generate
plenti-CaMKII_F	GTCAAGCCGGTTCTCCG	External primers for all genes in the pLenti vector
plenti-CaMKII_R	GTTAAGAATACCAGTCAATCTTTCAC	
SpyTag_C1C2_F	GCCCACATCGTGATGGTGGACGCCTACAAGCCCACCAAGTCGACTGGCAGTGACG	*pLenti-CaMKII::SpyTag-C1C2-TS-mCherry/EYFP*
SpyTag_C1C2_R	TTGGTGGGCTTGTAGGCGTCCACCATCACGATGTGGGCGGCTCCTGCGCTGC	
pLenti-C1V1-3	GTTCGCATCCCCTTCTCCAAC	*pLenti-CaMKII::SNAP-tag-C1C2-TS-mCherry*
C1C2-NS-R	GCGCTTCATTTCGCAGTCTTTGTCGGCTCCTGCGCTGCCGGCCGCCAG	
C1C2-SNAP-NS-start	GAGCCGACAAAGACTGCGAAATGAAGCG	
C1C2-SNAP-NS-end	CTGCCAGTCGAACCCAGCCCAGGCTTGCCCAGTCTGTG	
SNAP-CIC2-NS-mid	GCCTGGGCTGGGTTCGACTGGCAGTGACGCGACGG	
WPRE-R	GTTAAGAATACCAGTCAATCTTTCAC	
TS_For	GAGCAGGATCACCAGCG	Primers specific to the TS sequence between opsin and marker
TS_Rev	CGCTGGTGATCCTGCTC	
C1C2_Spy_TagDA_F	GTGATGGTGGCCGCCTACAAGCCCACCAAGTCGACTGGCAGTGACG	*pLenti-CaMKII::SpyTag(DA)-C1C1-TS-mCherry*
C1C2_Spy_TagDA_R	GTAGGCGGCCACCATCACGATGTGGGCGGCTCCTGCGCTGC	
N_term_Tag_C1C2_2	CGGGGGATCCCCGGGTACCGGTAGGCCACCATGGCCCACATCGTGATGGTGGACG	*pLenti-CaMKII::SpyTag(0)-C1C1-TS-mCherry*
N_term_Tag_C1C2_1	CCCACATCGTGATGGTGGACGCCTACAAGCCCACCAAGTCGCGGAGGCCATGGC	
pGP_Gib_ChR2_F	GCTAGCGCTACCGGACTCAGATCTCGCCACCATGGACTATGGCGGCGCT	*pGP-CMV::ChR2-mCherry*
pGP_Gib_ChR2_R	ATGGCTGATTATGATCTAGAGTCGCGGCCGCTTACTTGTACAGCTCGTCCA	
ChR2_SpyTag_F	CACATCGTGATGGTGGACGCCTACAAGCCCACCAAGTGAGCGGCCGCGACTCTAG	*pGP-CMV::ChR2-mCherry-SpyTag*
ChR2_SpyTag_R	GGGCTTGTAGGCGTCCACCATCACGATGTGGGCCTTGTACAGCTCGTCCATG	
pGP-Gib_F	GTTTAGTGAACCGTCAGATCCGCTAGCGCTACCGG	External primers for all genes in the pGP vector
pGP-Gib_ R	CAAGTAAAACCTCTACAAATGTGGTATGGCTGATTATGATCTAGAG	
ReaChR_fwd	GCGGGGGATCCCCGGGTACCGGTAGGCCACCATGGTGAGCAGAAGACCCTGGCTGC	*pLenti-CAMKIIa::ReaChR-TS-mCherry-WPRE*
ReaChR_rev	CCGCGCTGCTCTCGTACTTATCTTCTTC	
pA-TS-mcherry-fwd	GATAAGTACGAGAGCAGCGCGGCCGCCAAGAGCAGGATCAC	
WPRE-rev	GTTAAGAATACCAGTCAATCTTTCAC	
CAMKIIa-fwd	GTT CGC ATC CCC TTC TCC AAC	
Spy-ReaChR-fwd	CATCGTGATGGTGGACGCCTACAAGCCCACCAAGAGCACCGGCAGCGACGCCAC	*pLenti-CAMKIIa::SpyTag-ReaChR-TS-mCherry-WPRE*
Spy-ReaChR-rev	GTAGGCGTCCACCATCACGATGTGGGCGGCGCCGGCGCTGCCGGCGGCC	
trkB-fwd	GTCGTGCCTGAGAGCGCAGTCGAGAAACCGGTGCCACCATGAGCC	*pLenti-hSyn1::TrkB-3xGS linker- mCherry-WPRE*
3xGS-trkB-rev	CGCCCTTGCTCACGCTACCGCTGCCGCTACCGCCCAGGATGTCCAGGTACACGG	

3xGS-mcherry-fwd	CTGGACATCCTGGGCGGTAGCGGCAGCGGTAGCGTG AGCAAGGGCGAGGAGGATAAC	
mcherry-rev	GATTATCGATAAGCTTGATATCGAATTCTCATTACTTGT ACAGCTCGTCCATGCCGCC	
spy-trkB-rev	CACATCGTGATGGTGGACGCCTACAAGCCCACCAAGG GCGGTAGCGGCTGCCCCATGAGCTGCAAGTGCAG	*pLenti-hSyn1::SpyTag-TrkB-mCherry-WPRE*
spy-trkB-fwd	GATCCGGGTGGTGCTGCACTTGCAGCTCATGGGGCAG CCGCTACCGCCCTTGGTGGGCTTGTAGGCGTCCACCA TCACGATGTGGGCGGCCAGGCTGGCCCGCCAGAAGC	
hsyn-fwd	CAGGGACAGCAGAGATCCAGTTTGGTTAATTAAGTGTC TAGACTGCAGAGGGCCCTGCG	
Worm1	CCCTGCAGCAGTTTATCAGTTATCAGCAAGCAG	*hlh-12::SpyTag-C1C2-mCherry*
Worm2	CCGGATCCTTTAATAAAATTGTGTAAGATGACGC	
Worm3	GCGGTACCATGTCGCGGAGGCCATGGCTTCTT	
Worm4	GCGGTACCTCACTTGTACAGCTCGTCCATGCC	
Worm5	CGGGTACCATGCGGAAAATGCTACTTTTTTGC	
Worm6	CGGGTACCTCATGTGTGTCGAATCATTGGTTC	
Worm7	CGGGTACCATGAAAGGCAGCAGCCATCATCAT	*HS::lin-3::SpyCatcher-GFP*
Worm8	CGGGTACCTTATTTGTAGAGCTCATCGATGCC	
Worm9	GACCATGATTACGCCAAGCTTGCA	
Worm10	TGAAACTGTAAATTGAGGCATAAAGAGTAG	
Worm11	TTTATGCCTCAATTTACAGTTTCAATGAAAGGCAGCAG CCATCATCAT	
Worm12	ATCACCGAAACGCGCGAGACGAAA	
Worm13	CGGGGTACCATGGTGAGCAGAAGACCCTGGCTG	*unc-47::SpyTag-ReaChR-TS-mCherry*
Worm14	CCAGAGGTTGATTATCGATAAGC	
Worm15	CGGGCCGGCCATGTTGTCATCACTTCAAACTT	
Worm16	CGGGCGCGCCCTGTAATGAAATAAATGTGACG	
Worm17	TTTATGCCTCAATTTACAGTTTCAATGAAAGGCAGCAG CCATCATCAT	*pSM-HS::lin-3::SpyCatcher-GFP*
Worm18	CGGGTACCATGCGGAAAATGCTACTTTTTTGCATCCTT CTACTCTTTATGCCTCAATTT	
Worm19	CGGAATTCTTATTTGTAGAGCTCATCGATGCC	
Worm20	CGGGCCGGCCGACCATGATTACGCCAAGCTTGCA	
Worm21	CGGGCGCGCCGATCCGATGAGGATTTTCGAAGTT	
ReaCh_E130_NDT_F	CGCTGATTNDTATGATGAAAAGC	Primers for site-saturation at position E130
ReaCh_E130_VHG_F	CGCTGATTVHGATGATGAAAAGC	
ReaCh_E130_TGG_F	CGCTGATTTGGATGATGAAAAGC	
ReaCh_E130_AHN_R	CATCATAHNAATCAGCGCCACATA	
ReaCh_E130_CDB_R	CATCATCDBAATCAGCGCCACATA	
ReaCh_E130_CCA_R	CATCATCCAAATCAGCGCCACATA	
ReaCh_N298_NDT_F	CGAAGNDTATGTGGGGC	Primers for site-saturation at position N298
ReaCh_N298_VHG_F	CGAAGVHGATGTGGGGC	
ReaCh_N298_TGG_F	CGAAGTGGATGTGGGGC	
ReaCh_N298_AHN_R	ACATAHNCTTCGCAATCAGATC	
ReaCh_N298_CDB_R	ACATCDBCTTCGCAATCAGATC	
ReaCh_N298_CCA_R	ACATCCACTTCGCAATCAGATC	

Highlighted text is to indicate degenerate nucleotides for site-saturation library

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

SpyTag/SpyCatcher & SNAP-tag fusion constructs

The mammalian codon optimized *SpyTag* was first introduced into the N-terminus of *pLenti-CaMKIIa-C1C2-TS-EYFP* (**Table S2**) after the signal peptide cleavage site (between amino acid position 23 and 24 in the C1C2 sequence) by overlap extension PCR using external primers plenti-CaMKII_F and plenti-CaMKII_R, and internal primers SpyTag_C1C2_F and SpyTag_C1C2_R (**Table S3**). To generate the *pLenti-CMV/CaMKIIa::SpyTag-C1C2-TS-EYFP* (**Table S1**) the assembly product was then inserted into the *BamHI*/*EcoRI* cut *pLenti-CaMKIIa-C1C2-TS-EYFP* vector (**Table S2**). The *pLenti-CMV/CaMKIIa::SpyTag-C1C2-mCherry* construct (**Table S1**) was built by first amplifying *SpyTag-C1C2-TS* from the *pLenti-CMV/CamMKIIa-SpyTag-C1C2-TS-EYFP* (**Table S1**) construct using the plenti-CaMKII_F and TS_Rev primers (**Table S3**) and amplifying the *TS-mCherry* from *pAAV-CaMKII-C1V1-TS-mCherry* (**Table S2**) using TS_For and plenti-CaMKII_R (**Table S3**). The fragments were then assembled using overlap extension PCR with plenti-CaMKII_F and plenti-CaMKII_R primers (**Table S3**). The assembly product was then inserted into *BamHI*/*EcoRI* cut *pLenti-CaMKIIa-C1C2-TS-EYFP* (**Table S2**) vector using Gibson assembly. A similar process was used to generate the *pLenti-CaMKIIa::C1C2-mCherry* construct only the initial amplification was done using the *pLenti-CamMKIIa::C1C2-TS-EYFP* backbone. Note that all vectors denoted as having a *CaMKII*, *CaMKIIa* or *hSyn1* promoter also have an upstream *CMV* promoter. For the construct built for this work we have labeled the promoter as *CMV/CaMKIIa* since both promoters are present. The *CMV* promoter drives expression in transfections while the *CamKIIa* promoter would drive expression upon viral infection. These constructs can be used for both transfection of viral production and infection.

The mammalian codon optimized *SNAP-tag* sequence was first introduced into the N-terminus of *pLenti-CaMKIIa-C1C2-TS-mCherry* (**Table S2**) after the signal peptide cleavage site (between amino acid position 23 and 24 in the C1C2 sequence). The SNAP-tag sequence was amplified from $pSNAP_f$ vector (NEB, cat N9183S) with primers: C1C2-SNAP-NS-start and C1C2-SNAP-NS-end, and fused to C1C2 and mCherry with internal primers C1C2-NS-R and SNAP-CIC2-NS-mid, and external primers plenti-C1V1-3 and WPRE-R by overlap extension PCR method (**Table S3**). To generate the *pLenti-CMV/CaMKIIa::SNAP-tag-C1C2-TS-mCherry* (**Table S1**) the assembly product was then inserted into the *BamHI*/*EcoRI* cut *pLenti-CaMKIIa-C1C2-TS-mCherry* vector using Gibson assembly method (**Table S2**).

Substitution of the aspartic acid, the reactive residue in the SpyTag, to the non-reactive alanine was done through mutation of the codon from *GAC* to *GCC*. This mutation was introduced through overlap extension PCR. The *SpyTag-C1C2-mCherry* was amplified into two separate fragments with the mutation introduced at the beginning of one fragment and the end of the other fragment using the C1C2_Spy_TagDA_F/plenti-CaMKII_R and plenti-CaMKII_F/C1C2_Spy_TagDA_R primer pairs (**Table S3**). These fragments were assembled though PCR, digested with *BamHI*/*EcoRI* and then ligated into the *BamHI*/*EcoRI* cut *pLenti-CaMKIIa-C1C2-TS-EYFP* vector (**Table S3**) to produce the *pLenti-CMV/CaMKIIa::SpyTag(DA)-C1C1-TS-mCherry* construct.

To generate SpyTag-ChR2-mCherry variants *ChR2-mCherry* was amplified from *pLenti-CaMKIIa-hChR2(H134R)-mCherry-WPRE* (**Table S2**) using pGP_Gib_ChR2_F and pGP_Gib_ChR2_R primers (**Table S3**). Gibson assembly method was then used to insert the *ChR2-mCherry* amplification product into the *pGP-CMV-GCaMP6f* vector (**Table S2**) cut with *BglII/XbaI*. This produced the *pGP-CMV::ChR2-mCherry* construct that was then used for all ChR2, SpyTag fusions. The C-terminal fusion of *SpyTag* to *ChR2-mCherry* (*pGP-CMV::ChR2-mCherry-SpyTag*) was generated by overlap-extension PCR by first amplifying the *ChR2-mCherry-SpyTag* in two parts with ChR2_SpyTag_F/ pGP-Gib_ R and pGP-Gib_ F/ ChR2_SpyTag_R primer pairs. The two amplified fragments were then assembled using ChR2_SpyTag_F/ ChR2_SpyTag_R primers. The assembly product was inserted into the *pGP-CMV-GCaMP6f* vector (**Table S2**) cut with *BglII/XbaI*. Stepping of the SpyTag at the N-terminal end of ChR2 was done using the same method using different SpyTag insertion primers labeled as SpyTag_ChR2_# based on the position described in **Figure S4**.

ReaChR rhodopsin was fused to the mCherry reporter after a three-alanine residue linker, and a trafficking signal (TS) KSRITSEGEYIPLDQIDINV (Gradinaru et al., 2010). The *ReaChR* gene was amplified from *AAV-EFIa-ReaChR-mCitrine-FLEX* vector (**Table S2**) using ReaChR_fwd and ReaChR_rev primers (**Table S3**). The *3xA-linker-TS-mCherry* was amplified from *pLenti-CAMKIIa-C1C2-TS-mCherry* (**Table S1**) plasmid using pA_TS_mcherry_fwd and WPRE_rev primers (**Table S3**). *ReaChR-TS-mCherry* was assembled using overlapping assembly PCR and digested with *EcoRI* and *BamHI*. Digested insert was ligated into an *EcoRI*/*BamHI* digested Lentiviral vector containing the *CMV*/*CaMKIIa* promoters and WPRE to obtain the *pLenti-CMV/CaMKIIa::ReaChR-TS-mCherry-WPRE* clone (**Table S1**). *SpyTag* was inserted at the N-terminus of *ReaChR* after the signal peptide cleavage site (24 amino acids from the N-terminus) using overlap extension

PCR with primers Spy_ReaChR_fwd, Spy_ReaChR_rev, WPRE_rev and CAMKIIa_fwd (**Table S3**). Digestion and ligation of the assembled product into the template lentiviral vector produced the *pLenti-CMV/CaMKIIa::SpyTag-ReaChR-TS-mCherry-WPRE* clone (**Table S1**).

The *pLenti-CMV/hSyn1::TrkB-mCherry-WPRE* vector (**Table S1**) was built by Gibson assembly method. A lentiviral vector containing *human synapsin I* (*hSyn1*) promoter and *WPRE*, *pLenti-hSyn-eNpHR 3.0-EYFP* (**Table S2**), was digested with *AgeI* and *EcoRI* enzymes and used as backbone for all TrkB constructs. *TrkB* was synthesized from GenScript USA Inc, fused with *EYFP* reporter and inserted into this lentiviral vector to build *pLenti-CMV/hSyn1::TrkB-EYFP-WPRE* vector. To replace the *EYFP* marker with *mCherry* the *mCherry* gene from *pLenti-CaMKIIa-ReaChR-TS-mCherry-WPRE* vector was amplified with 3xGS_mcherry_fwd and mcherry_rev primers (**Table S3**). Assembly product of the *TrkB*, *mCherry* fusion was generated using TrkB_fwd and 3xGS_TrkB_rev primers, and then inserted into the digested lentiviral vector. *pLenti-CMV/hSyn1*::SpyTag-TrkB-mCherry-WPRE* (**Table S1**) was built by inserting the *SpyTag-GGSG* linker at the N-terminus of *TrkB* after the signal peptide cleavage site (31 amino acids from the N-terminus) using the overlapping primers spy_trkB_rev and spy_trkB_fwd, and assembled with end primers hsyn_fwd and mcherry_rev primers. This was then inserted into the template lentiviral vector containing *hSyn1* promoter at sites *AgeI* and *EcoRI* using Gibson assembly method.

To generate *C. elegans* expression plasmid *hlh-12::SpyTag-C1C2-mCherry*, 1.2 kb of the *hlh-12* 5' region was PCR amplified from genomic DNA using primers Worm1 and Worm2, and cloned into pPD49.26 (Fire vector) using *PstI* and *BamHI* restriction sites. Then, the *SpyTag-C1C2-mCherry* sequence was PCR amplified from plasmid *pLenti-CaMKIIa::SpyTag-C1C2-mCherry* (**Table S1**) using primers Worm3 and Worm4, and was cloned into ppD49.26 *hlh-12* vector using the *KpnI* restriction site. Plasmid pSM*::unc-47::SpyTag-ReaChR-TS-mCherry* was generated by first PCR amplifying *SpyTag-ReaChR-TS-mCherry* from *pLenti-CaMKIIa::SpyTag-ReaChR-TS-mCherry* using primers Worm13 and Worm14 and inserting the PCR product into vector pSM::GFP (gift from Cori Bargmann) using *KpnI* and *EcoRI* restriction sites. pSM*::unc-47::SpyTag-ReaChR(E130D)-TS-mCherry* was constructed in the same way only by PCR amplifying *SpyTag-ReaChR(E130D)-TS-mCherry* from *pLenti-CaMKIIa::SpyTag-ReaChR(E130D)-TS-mCherry*. 1.5 kb of the *unc-47* 5' region was PCR amplified from genomic DNA using primers Worm15 and Worm16, and cloned into pSM::*SpyTag-ReaChR-TS-mCherry* using *FseI* and *AscI* restriction sites (**Table S1**).

PCR fusion product *HS::lin-3 signal sequence::SpyCatcher-GFP* was constructed by PCR fusion of PCR products from a *HS::lin-3* plasmid and *SpyCatcher-GFP* plasmid. To generate *HS::lin-3* plasmid, a partial *lin-3* (*C. elegans* EGF) sequence was PCR amplified from genomic DNA using primers Worm5 and Worm6, and inserted into plasmid *pPD49.83* (Fire vector containing HS promoter) using the *KpnI* restriction site. *SpyCatcher-GFP* plasmid was generated by amplifying *SpyCatcher-GFP* sequence from plasmid *pQE80l-T5::6xhis-SpyCatcher-Elp-GFP* using primers Worm7 and Worm8 and inserting into plasmid pPD49.83 using the *KpnI* restriction site. *HS::lin-3 signal sequence* was PCR amplified from the *HS::lin-3* plasmid using primers Worm9 and Worm10, and *SpyCatcher-GFP::unc-54 3' UTR* sequence was amplified from *SpyCatcher-GFP* plasmid using primers Worm11 (containing overlap to the *lin-3* signal sequence) and Worm12. *HS::lin-3 signal sequence* and *SpyCatcher-GFP::unc-54 3' UTR* PCR products were fused through a second PCR reaction using both products as templates and primers Worm9 and Worm12.

Plasmid *pSM-lin-3 signal sequence::SpyCatcher-GFP::unc-54 3'UTR* was generated by adding on the *lin-3 signal sequence* to *SpyCatcher-GFP::unc-54 3'UTR* through two PCR reactions, first using primers Worm17 and Worm19 and *SpyCatcher-GFP* plasmid as template. The PCR product was then amplified using primers Worm18 and Worm19. The product was cloned into *pSM-GFP* vector using the *KpnI* and *EcoRI* restriction sites. *HS* sequence was PCR amplified from *pPD49.83* using primers Worm20 and Worm21 and the product was cloned into *pSM-lin-3 signal sequence::SpyCatcher-GFP::unc-54 3'UTR* using *FseI* and *AscI* restriction sites to generate the final plasmid, *pSM-HS::lin-3 signal sequence::SpyCatcher-GFP::unc-54 3'UTR.*

To build the *pQE80l-T5::6xhis-SpyCatcher-Elp-GFP* construct new restriction sites were introduced for our system (for details see Sun *et al*.). The original restriction sites following His6 tag in pQE-80l were removed. This SpyCatcher-GFP construct was derived from SpyCatcher-Elp-SpyCatcher (pQE-BB) described in Sun *et al.* (Sun et al., 2014). The GFP gene with a TAA stop codon was inserted between *SacI* and *SpeI* sites to generate the final construct.

Generating site-saturation library from the Tag-ReaChR-mCherry parent

Primers designs are listed in **Table S3** with degenerate residues highlighted in yellow. Mutations were introduced by overlap extension PCR of the *pLenti-CaMKIIa::SpyTag-ReaChR-TS-mCherry* parent backbone

with external primers plenti-CaMKII_F/ plenti-CaMKII_R used for amplification and assembly. Assembly product was then digested with *EcoRI/BamHI* and ligated into *EcoRI/BamHI* cut *CaMKIIa::SpyTag-ReaChR-TS-mCherry* vector. Each library was then transformed into *E. coli*, single colonies were picked and 2-5 ml cultures were grown for each variant. DNA for each variant was purified and the concentration of DNA for each variant was normalized to 100 ng/ul for transfection into HEK cells.

HEK cell maintenance and transfection

HEK 293F cell were cultured at 37 ^{o}C and 5% CO2 in D10 (Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FBS, 1% sodium bicarbonate, 1% sodium pyruvate, and penicillin-streptomycin). For low throughput confocal imaging constructs were transfected with Fugene6 into HEK cells according to the manufacturer's protocol plated at a density of 5,000 cells per cm^2 onto 12 mm- PolyDLysine coated coverslips at 18 hours post-transfection. The HEK cells were then left to adhere to coverslips and continue to express for another 30 hours (so total expression for 48 hours post transfection) before labeling with SpyCatcher and imaging. For the 96-well format screening HEK cells were seeded at low density in tissue culture treated 96-well plates (BD Falcon Microtest^{TM} 96). Cells were left to divide until they reached ~20-30% confluency. Library variants were transfected with Fugene6 into HEK cells according to the manufacturer's recommendations with one variant per well (with pre-normalized DNA concentration of each variant). Cells were then labeled with SpyCatcher 48 hours post transfection and imaged.

Preparation and transfection of primary neuronal cultures

Rat hippocampal cells were dissected from Wistar embryos (prenatal days E18, Charles-River Labs), and cultured at 37oC, 5% CO2 in Neurobasal media supplemented with B27, glutamine, and 2.5% FBS. 3 days after plating, glial growth was inhibited by addition of FUDR. Cells were transfected 4-5 days after plating with SpyTag-opsin variants using calcium chloride. Neurons were labeled with SpyCatcher and imaged 2-5 days after transfection.

SpyCatcher labeling of HEK cells in 96-well format

SpyCatcher labeling protocol for 96-well plate. To avoid any variability in labeling in the 96-well format screen a saturating concentration of the SpyCatcher (30 μM) was used for labeling experiments. A 75 μM SpyCatcher stock was made and 20 μl of the stock was added to 30 μl of D10 in each well for a final concentration of 30 μM SpyCatcher per well. The cells were then incubated with the labeling protein at room temperature for 45 minutes. After the labeling the cells were washed. To avoid complete removal of media from the cells 200 μl of fresh D10 was added to each well to dilute the SpyCatcher concentration and then 200 μl was removed from each well. This washing/dilution was repeated four times. After washing the 96-well plates of cells were returned to a 37 ^{o}C incubator and left for 30 minutes before imaging. For imaging of cells in each well the media was replaced with extracellular buffer (in mM: 140 NaCl, 5 KCl, 10 HEPES, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose; pH 7.35) to avoid the high autofluorecence of the D10.

SNAP labeling of HEK cells

SNAP-Surface® 488 was purchased from NEB (cat S9124S). Labeling of live HEK cells transfected with *pLenti-CaMKII::SNAP-tag-C1C2-TS-mCherry* was done following manufacturer's instructions for cellular labeling. In brief, the SNAP-Surface® 488 reconstituted in DMSO to make a 1 mM stock solution. The stock solution was then diluted 1:200 in D10 media to yield a labeling medium of 5 uM dye substrate. The SNAP-tag-C1C2-mCherry expressing HEK cells were then incubated in the labeling medium for 30 min at 37^{o}C. After labeling the cells were washed 3-4x with D10 media before confocal imaging.

Generating and maintaining SpyTag and SpyCatcher transgenic *C. elegans* strains

C. elegans strains were cultured at room temperature using standard protocols unless indicated otherwise (Brenner, 1974). Strains used in this study were *him-5*(*e1490*) (Hodgkin et al., 1979) and *unc-119(ed4)* (Maduro and Pilgrim, 1995). Transgenic *C. elegans* expressing Tag-C1C2-mCherry was generated by co-injecting plasmid *hlh-12::SpyTag-C1C2-mCherry* (14 ng), *unc-119* rescue plasmid (60 ng), and 1kb ladder carrier DNA (50ng) into *unc-119* mutant animals. A transgenic *C. elegans* line expressing heat-shock activated Catcher-GFP and specific expression of Tag-C1C2-mCherry in DTCs was generated by co-injecting plasmid *hlh-12::SpyTag-C1C2-mCherry* (14 ng), PCR fusion product *HS::lin-3 signal sequence::SpyCatcher-GFP* (40 ng), 1kb ladder carrier DNA (50 ng), and *unc-119* rescue plasmid (60 ng), into *unc-119* mutant

animals. Transgenic animals expressing heat-shock activated Catcher-GFP and either wild-type or mutant *SpyTag-ReaChR-TS-mCherry* in GABA neurons was generated by co-injecting plasmid *unc-47::SpyTag-ReaChR-TS-mCherry* (wild-type or mutant 90 ng), plasmid *HS::lin-3 signal sequence::SpyCatcher-GFP* (50 ng), 1kb ladder carrier DNA (50 ng), and *unc-119* rescue plasmid (60 ng), into *unc-119* mutant animals.

SpyCatcher staining of dissected *C. elegans* gonad

To extrude gonads from animals, hermaphrodites were placed in 6 mL of PBS (phosphate buffered saline) on a Superfrost plus microscope slide (Fisher Scientific) and cut below the pharynx with a razor blade as described previously (Chan and Meyer, 2006). 6 mL of 4% p-formaldehyde solution was added, sandwiched with a coverslip, and fixed for 10 minutes. The entire slide was then submersed in liquid nitrogen for a few minutes, and immediately upon removal, the coverslip was removed and the slide was washed with PBS three times. 30 mL of purified Catcher-GFP in PBS solution (20 μM) was applied to the fixed gonads on the slide and incubated for 30 minutes at room temperature. The slide was washed 3x5 minutes with PBS and imaged after mounting with Vectashield mounting media (Vector Laboratories).

Heat-shock treatment to induce SpyCatcher expression

C. elegans strain carrying transgenes *hlh-12::SpyTag-C1C2-mCherry* and *HS::lin-3 signal sequence::SpyCatcher-GFP* was heat-shock treated at 33°C for 15 minutes in a water bath. *C. elegans* strain carrying transgenes *HS::lin-3 signal sequence::SpyCatcher-GFP and either wild-type or mutant unc-47::SpyTag-ReaChR-TS-mCherry* were heat-shock treated at 33°C for 30 minutes. Following heat-shock, animals were allowed to recover at room temperature. At specific time points they were placed on an agar pad in 3 mM levamisole and imaged.

Locomotion assay evoked by green light

Animals expressing either wild-type or mutant *unc-47::SpyTag-ReaChR-TS-mCherry* were grown on NGM plates with OP50 bacteria and all-trans retinal. 150μL of OP50 culture alone or with 100μM all trans-retinal (0.15μL of 100mM stock in ethanol; Sigma-Aldrich) was added to NGM plates and dried for several hours in the dark. L4-stage transgenic animals were placed on plates and grown in the dark for approximately

16 hours. To assay paralysis, animals were transferred individually onto plain NGM plates and their movement was monitored on a dissecting microscope (Leica) at 2.5x magnification for 10 s without green light, 5 s with green light illumination, and 10 s without green light. Green light (650±13 nm) was generated using LED illumination using a Lumencor SPECTRAX light engine at a power of 1 mW. White light illumination, which was constant throughout the experiment, was filtered to remove blue/green light. Paralysis upon illumination was scored as a positive.

Fluorescence Imaging

For non-confocal imaging of cultured neurons expressing different opsin variants a Zeiss Axio Examiner.D1 microscope with a 20x 1.0 NA water immersion objective (Zeiss W Plan Apochromat 20x/1.0 DIC D=0.17 M27 75mm) was used. Images of neurons were taken before electrophysiological recordings and the images we analyzed for fluorescence level comparison between variants. Imaging of the mCherry fusion fluorescence was excited with 650±13 nm, and imaging of the GFP label fluorescence was excited with 485±20 nm. Both wavelengths of light were generated with LED illumination using a Lumencor SPECTRAX light engine with quad band 387/485/559/649 nm excitation filter, quad band 410/504/582/669 nm dichroic mirror and quad band 440/521/607/700 nm emission filter (all SEMROCK).

Confocal imaging was preformed on a Zeiss LSM 780 Confocal Microscope. Imaging of live cultured HEK cells and neurons was preformed with a Zeiss W Plan-APOCHROMAT 20x/1.0 DIC(UV) Vis-IR objective. Imaging of live *C. elegans* was preformed using a Zeiss LD LCI Plan-APOCHROMAT 25x/0.8 Imm Korr DIC M27 objective. GFP fluorescence was excited with a 488 nm laser and mCherry fluorescence was excited with a 561 nm laser. Fluorescence emission was imaged using the LSM 780's GaAsP detectors with a detection range of 499-606 nm for GFP and 578-695 nm mCherry. Imaging was done with excitation and emission measurements of GFP and mCherry done on separate tracks to avoid crossover. Imaging settings were matched across experiments to enable comparison.

Full population images of cells in 96-well plates were taken with a Leica DM IRB microscope and the Leica microsystems objective HC PL FL 10x/0.30 PH1. Cells were illuminated with LEJ ebq 50 ac mercury lamp. GFP fluorescence was imaged with SEMROCK Blue light filter set: SEMROCK BrightLine® single-band filter set with BrightLine® single-band bandpass excitation filter (482/18 nm), emission filter (520/28) and 495

nm edge BrightLine® single-edge dichroic beamsplitter. mCherry fluorescence was imaged with Leica's N2.1 filter cube with bandpass excitation filter (515-560 nm), longpass suppression filter (590 nm) and dichromatic mirror (580).

Electrophysiology

Conventional whole-cell patch-clamp recordings were done in cultured HEK cells and cultured rat hippocampal neurons at least 2 days post transfection. Cells were continuously perfused with extracellular solution at room temperature (in mM: 140 NaCl, 5 KCl, 10 HEPES, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose; pH 7.35) while mounted on the microscope stage. Patch pipettes were fabricated from borosilicate capillary glass tubing (1B150-4; World Precision Instruments, Inc., Sarasota. FL) using a model P-2000 laser puller (Sutter Instruments) to resistances of 2-5 MΩ. Pipettes were filled with intracellular solution (in mM): 134 K gluconate, 5 EGTA, 10 HEPES, 2 $MgCl_2$, 0.5 $CaCl_2$, 3 ATP, 0.2 GTP. Whole-cell patch-clamp recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), a Digidata 1440 digitizer (Molecular Devices), and a PC running pClamp (version 10.4) software (Molecular Devices) to generate current injection waveforms and to record voltage and current traces.

Patch-clamp recordings were done with short light pulses to measure photocurrents. Photocurrents induced by green light were measured using 590±25 nm LED at 1 mW mm^{-2} for ReaChR-mCherry and Tag-ReaChR-mCherry variants. While C1C2-mCherry and Tag-C1C2-mCherry variant's photocurrents were induced by cyan light were measured using 485±20 nm LED at 0.3 mW mm^{-2}. Photocurrents were recorded from cells in voltage clamp held at -50 mV with 3-10 light pulse trains (0.5 s each pulse; 2 s apart). Both wavelengths were produced using LED illumination using a Lumencor SPECTRAX light engine with quad band 387/485/559/649 nm excitation filter, quad band 410/504/582/669 nm dichroic mirror and quad band 440/521/607/700 nm emission filter (all SEMROCK).

Action spectra measurements were performed for the following wavelengths: 386±23 nm, 485±20 nm, 590±25 nm, and 650±13 nm with light intensity matched across all experiments at 0.1 mW mm^{-2}. Each light pulse was delivered for 0.6 s with 10 s breaks between light pulses. All wavelengths were produced using LED illumination from a SPECTRAX light engine (Lumencor). Cell health was monitored through holding current and input resistance.

Data analysis

A MATLAB script was written to compare area above a threshold level of fluorescence in a population of cells. This was done for both mCherry fluorescence and GFP fluorescence. The mCherry-above-threshold-area was then used to normalize the GFP-above-threshold-area so that the density of cells within the image was not a confounding factor. The ratio of GFP-above-threshold-area to mCherry-above-threshold-area was the metric used to compare across the libraries reported in **Figure 3**.

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